REMARKS

1. Disposition of Claims

Claims 16 and 17 are pending in this application. Claims 15, 19, and 23-25 have been canceled, and Claim 17 (the independent claim) has been amended, all in the interest of accelerated prosecution, and thus for reasons unrelated to patentability. The enablement rejection has been maintained. The obviousness rejection is appreciated as being withdrawn. No new matter has been added. Reexamination and reconsideration of the application, as amended, are respectfully requested.

2. Compliance with 35 USC 112/1 - Enablement

The Patent Office rejected the claims under 35 USC 112/1 as failing to meet the enablement requirement on the reasoning that, while the Office agrees with Applicant that one skilled in the art could attach PEG to a phage, there is no evidence that the PEGylated phage would possess the claimed property – i.e., delayed inactivation by the host defense system. Office Action dated 12/13/2005, page 5 ("There are no specific teachings in the disclosure that would allow one to have a reasonable expectation of success in practicing the claimed invention except by the application of PEG."). Office Action dated 06/07/2006, page 3 ("One would expect that PEG can be chemically conjugated to bacteriophages and that this would protect the phages from immune clearance."). Office Action dated 11/28/2006, page 6 ("In summary, while the Office agrees with Applicant that one skilled in the art could attach PEG to a phage, there is no evidence that the PEGylated phage would possess the claimed property – i.e., delayed inactivation by the Host Defense System."). Under MPEP 2138.05, the filing of a patent application is a constructive reduction to practice and thus an actual reduction to practice need not be shown. Nevertheless, the property of delayed inactivation of PEGylated bacteriophage by an animal's host defense system has been struck from the claims, leaving the claims defining a PEGylated bacteriophage as an intermediate or starting material.

Under MPEP 806.05(j), an intermediate constitutes patentable subject matter, where by "intermediate" is meant an intermediate or starting product that may or may not be mutually exclusive with a final product. Here, a PEGylated bacteriophage is an "intermediate" that is meant to be an intermediate or starting product that may or may not be mutually exclusive with a final product, a PEGylated bacteriophage that is able to delay inactivation by an animal's host

defense system. Additionally, here, one process by which the "intermediate" (a PEGylated bacteriophage) may be demonstrated to be co-extensive with a final product (a PEGylated bacteriophage that is able to delay inactivation by an animal's host defense system) is provided in Example 3 of the patent specification describing how to test for a PEGylated bacteriophage that retains infectivity and possesses increased half-life. Because a PEGylated bacteriophage as an intermediate or starting material is agreed by the Patent Office to be enabled and because the subject matter constitutes patentable subject matter, the conclusion is Claim 17 (and Claim 16 dependent thereon) defining a PEGylated bacteriophage is in compliance with 35 USC 112/1 as meeting the enablement requirement.

3. Compliance with 35 USC 112/1 – SC-MPEG

In O'Riordan et al. Hum Gene Ther 10: 1349 (May 1999), of record, covalent attachment of PEG to the surface of adenovirus by coupling PEG with tresyl-MPEG called TMPEG was reported to have preserved infectivity while reducing antigenicity. While it is true that use of other coupling methods decreased the infectivity of adenovirus (i.e., coupling PEG with cyanuric chloride-activated MPEG called "CC-MPEG" and coupling PEG with succinimidyl succinate activated MPEG called "SS-MPEG"), Applicant's preferred and exemplified method in Example 1 coupled PEG with succinimidyl carbonate activated MPEG termed "SC-MPEG". Applicant's preferred and exemplified method using SC-MPEG was not tested by the O'Riordan investigators. Additionally, the PEGylation method using TMPEG was published as Delgado et al., Biotechnol Appl Biochem 12: 119 (1990), of record. In short, not only did the O'Riordan paper not disparage Applicant's preferred and exemplified method using SC-MPEG but also it illustrated that PEGylated phage could have been prepared using O'Riordan's favored TMPEG method because it was published in 1990. The Patent Office takes the position that the chemistry of succinimidyl succinate and succinimidyl carbonate in activating PEG is the same or similar, thus Applicant's preferred method of activating PEG and PEGylating phage would result in reduced infectivity. As described in the pre-filing date art of Zalipsky et al., Biotechnol Appl Biochem. 15: 100-114 (1992), attached, however, these chemistries are not the same. Compared to the conventionally used SS-PEG, the reagent, SC-PEG, is slightly less reactive yet more selective. Additionally, the conjugates obtained through the use of SC-PEG were chemically more stable than SS-PEG conjugates. In any event, the activation of PEG to produce minimal

loss of infectivity of the virus can be optimized as demonstrated in the post-filing date art of Croyle et al., Hum Gene Ther. 11:1713-1722 (2000), attached.

CONCLUSION

Applicant respectfully requests that a timely Notice of Allowance be issued in this case. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the below-given telephone number.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 8/3/07

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AMEND

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BIOTECHNOLOGY AND APPLIED BIOCHEMISTRY 15, 100–114 (1992)

Evaluation of a New Reagent for Covalent Attachment of Polyethylene Glycol to Proteins

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ZALIPSKY, S., SELTZER, R., AND MENON-RUDOLPH, S. Evaluation of a New Reagent for Covalent Attachment of Polyethylene Glycol to Proteins. *Biotechnol. Appl. Biochem.* 15, 100–114 (1992).

Methoxypolyethylene glycol of molecular weight 5000 was converted to a reactive succinimidyl carbonate form (SC-PEG). The usefulness of this new polymeric reagent for the covalent attachment of polyethylene glycol to proteins was evaluated. SC-PEG was found to be sufficiently reactive to produce extensively modified proteins under mild conditions within 30 min, showing the highest reactivity around pH 9.3. The commonly used succinimidyl succinate derivative of methoxypolyethylene glycol (SS-PEG) served as a reference standard to which the new reagent was compared. The stability of the polymer–protein linkages, studied on a series of PEG-modified bovine serum albumins, provided the single most important difference between the two activated polymers. Urethane-linked PEG-proteins obtained through the use of SC-PEG showed considerably higher chemical stability than SS-PEG-derived conjugates. The measured rate constants of aminolysis (using N^{α}-acetyllysine) and hydrolysis showed that SC-PEG is slightly less reactive yet more selective of the two reagents. Hydrolysis of the active groups on SC-PEG was on average twofold slower than that on SS-PEG. The differences in the rates of aminolysis were even smaller than those in hydrolysis. PEG-trypsin conjugates produced by both activated polymers showed similar properties: they had no proteolytic activity, well-preserved esterolytic activity, and enhanced activity toward *p*-nitroanilide substrates. Michaelis–Menten constants of the modified enzymes were determined using N^{α}-benzyloxycarbonyl-L-arginine *p*-nitroanilide. These measurements indicated that the attachment of PEG to trypsin caused an increase in both the rate of turnover of the substrate and its affinity toward the modified enzymes. Through a series of experiments involving the appropriate polymeric and low-molecular-weight model compounds, it was demonstrated that these increases in amidolytic activity were unrelated to tyrosyl residues acylation by either one of the activated polymers.

Since the first report of Davis and co-workers on polyethylene glycol (PEG)[2]-modified proteins (1), a great deal of interest has been generated in this type of macromolecular conjugates. For example, PEG-enzymes [chymotrypsin (2), papain (3), horseradish peroxidase (4), cholesterol oxidase (5), lipase (6)] have been shown to be soluble

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[2] Abbreviations used: PEG, polyethylene glycol; mPEG, monomethoxy-PEG; SC-PEG, succinimidyl carbonate-PEG; SS-PEG, succinimidyl succinate-PEG; PBS, phosphate-buffered saline; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; BAPA, N^{α}-benzoyl-DL-arginine *p*-nitroanilide; BSA, bovine serum albumin; GF-HPLC, gel filtration high-performance liquid chromatography; HOSu, *N*-hydroxysuccinimide; NAL, N^{α}-acetyllysine; NAT, N^{α}-acetyltyrosine; NATE, N^{α}-acetyltyrosine ethyl ester; TEA, triethylamine; TNBS, trinitrobenzenesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; ZAPA, N^{α}-benzyloxycarbonyl-L-arginine *p*-nitroanilide.

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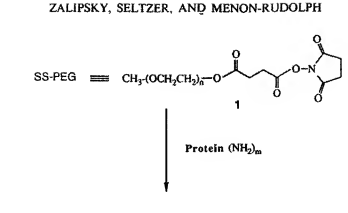
and active in organic solvents. Due to their affinity to the upper phase of PEG/dextran two-phase systems, PEG-modified proteins [concanavalin A (7), immunoglobulins (8), protein A (9)] have proved useful as diagnostic tools and as means for separating different types of biological cells. Undoubtedly, the most remarkable consequence of protein modification with PEG is the dramatically reduced immunogenicity and antigenicity of the conjugate. Also, PEG-protein conjugates, when injected into a living organism, were shown to remain in the bloodstream considerably longer than the corresponding native proteins. These last two characteristics of PEG-proteins were proven to be general, and allowed the development of a number of PEG-modified therapeutic proteins: enzymes [asparaginase (10), arginase (11), adenosine deaminase (12), superoxide dismutase (13, 14), catalase (15), tissue plasminogen activator (16), and others (17)], interleukin 2 (18), and hemoglobin (19). It was also demonstrated that PEG-modified potent allergen proteins can be useful in immunotherapy and tolerance induction (20).

To effect covalent attachment of the polymer to a protein, first the hydroxyl end groups of PEG have to be converted into reactive functional groups. This process is frequently referred to as "activation" and the product is called "activated PEG" (for a comprehensive review see Ref. (21)). In most cases the terminus of methoxypolyethylene glycol (mPEG) was capped with a functional group, to make it reactive toward amines on a protein molecule.

The cyanuric chloride approach for PEG activation introduced by Abuchowski *et al.* (1) suffers from drawbacks, such as toxicity of the reagent and excessive reactivity of the activated PEG toward functional groups other than amines (e.g., cysteine, tyrosine residues). Thus, the modification of some proteins with cyanuric chloride-activated PEG was often accompanied by substantial loss of biological activity (10, 21). To overcome these shortcomings several alternative methods of activation were introduced. The carbonyldiimidazole-activated PEG introduced by Pizzo and coworkers (13, 16) has rather mild reactivity and therefore long reaction times are required to achieve extensive protein modification (48–72 h). However, the proteins modified by this method usually exhibited good preservation of activities. Recently, Veronese *et al.* (14) reported the use of PEG-phenylcarbonate derivatives, which, similar to the carbonyldiimidazole-activated PEG, upon reaction with proteins yields urethane-linked PEG-proteins. The main drawback of this approach is in the toxicity of the hydrophobic phenol residues (*p*-nitrophenol or 2,4,5-trichlorophenol) and their affinity toward proteins. The succinimidyl succinate derivative of the polymer (SS-PEG, **I**) introduced by Abuchowski *et al.* (10) proved to be the most useful in our laboratory. It reacts fast (30 min) with proteins under mild conditions yielding active yet extensively modified conjugates (Scheme 1). One property of PEG-proteins obtained by the use of SS-PEG is noteworthy: the ester linkage between the polymer and the succinic acid residue has limited stability in aqueous media (20, 22).

It is apparent that each of the activated forms of the polymer has features which can be considered advantageous or disadvantageous, depending on the user's goals. In light of the many interesting applications of PEG-polypeptides, it is highly desirable to broaden the arsenal of protein modifying PEG derivatives tailor-made for a specific end use.

In this study we are reporting the properties of a new PEG-reagent for modification of proteins, namely succinimidyl carbonate of methoxypolyethylene glycol (SC-PEG, 2) (23). Special emphasis was given to the comparison of the new reagent with



SCHEME I. Attachment of PEG to a protein using SS-PEG as the activated form of the polymer.

conventionally used SS-PEG. Trypsin was modified as a model protein by both activated PEGs and the results were compared.

EXPERIMENTAL

General. All the chemical reagents were purchased at Aldrich or Fluka unless specified otherwise. Monomethoxy-PEG of molecular weight 5000 was a generous gift from Union Carbide Corp. SS-PEG was a product of Enzon (10). The proteins trypsin and bovine serum albumin, fraction V, were obtained from Boehringer-Mannheim and Sigma, respectively. Protein concentration was determined by Biuret assay (24). Kinetic and enzymatic measurements were performed on the Shimadzu UV-160 spectrophotometer. A Waters HPLC system equipped with uv and refractive index detectors was used for the analysis of PEG-modified proteins and for the measurements of mPEG-5000 release from PEG-BSA conjugates.

Preparation of SC-PEG (23). Warning! Synthesis of this reagent involves the use of phosgene, which is a highly toxic substance. All manipulations should therefore be performed in a well-ventilated hood. Methoxypolyethylene glycol (60 g, 12 mmol), dried by azeotropic removal of toluene, was dissolved in toluene/dichloromethane (3/1, 200 ml) and treated with a toluene solution of phosgene (30 ml, 57 mmol) overnight. The solution was evaporated to dryness and the remainder of phosgene was removed under vacuum. The residue was redissolved in toluene/dichloromethane (2/1, 150 ml) and treated with solid *N*-hydroxysuccinimide (2.1 g, 18 mmol) followed by triethylamine (1.7 ml, 12 mmol). After 3 h the solution was filtered and evaporated to dryness. The residue was dissolved in warm (50°C) ethyl acetate (600 ml), filtered from trace of insolubles, and cooled to facilitate precipitation of the polymer. The product was collected by filtration and then recrystallized once more from ethyl acetate. The yield was 52.5 g (85%). I.R. (film on NaCl, cm^{-1}) characteristic bands at: 1812 and 1789 (both C=O, succinimide); 1742 (C=O, carbonate); 1114 (CH_2OCH_2). ^{13}C NMR ($CDCl_3$): δ 168.5 ($CH_2\underline{C}$=O); 151.3 (O–CO_2); 71.9 ($CH_3O\underline{C}H_2$); 70.2 (PEG); 68.7 ($\underline{C}H_2CH_2OCO_2$); 68.0 ($CH_2\underline{C}H_2OCO_2$); 58.9 ($CH_3O$);

25.2 ($\underline{CH}_2C{=}O$) ppm. The active carbonate content of the product was determined according to Kalir *et al.* (25) by reacting aliquots of the polymer with an excess of benzylamine and back-titration of the latter with perchloric acid in dioxane using thymol blue as an indicator. These titrations indicated that 1 g of the product contained 1.97×10^{-4} mol of active carbonate (101% of theoretical value).

Determination of hydrolysis and aminolysis constants of the activated PEGs. All the measurements were performed by methods similar to those described by Whitesides and co-workers (26). For aminolysis experiments, to phosphate buffer (0.1 M, 1 ml) at the appropriate pH, a stock solution of N^{α}-acetyllysine (NAL) in water (30 mM, 0.1 ml) was added followed by a stock solution of the appropriate activated PEG in CH_3CN (1 mM active acyl, 0.1 ml). The resultant solution was quickly mixed and placed in a sample cuvette in the UV spectrophotometer and the appearance of *N*-hydroxysuccinimide anion ($^-$OSu) was monitored at 260 nm. For hydrolysis experiments, water was substituted for the NAL solution. The constants were calculated as follows: the first-order hydrolysis constant $K_h = \text{Rate}_h/[\text{SX-PEG}]_0$, where $\text{Rate}_h = dA_{260}/dt \times 1/\epsilon_{260} \times 1/F$; $[\text{SX-PEG}]_0$ = concentration of PEG-bound succinimidyl active acyl; $\epsilon_{260} = 8500\ \text{M}^{-1} \cdot \text{cm}^{-1}$ is the extinction coefficient of $^-$OSu; and $F = [^-\text{OSu}]/\{[\text{HOSu}] + [^-\text{OSu}]\} = (1 + 10^{6.0-\text{pH}})^{-1}$. The first-order aminolysis constant $K_{am} = \text{Total Rate}/[\text{SX-PEG}]_0 - K_h$. Total Rate in aminolysis experiments was calculated the same way as Rate_h in hydrolysis experiments.

Preparations of PEG-trypsin conjugates. The appropriate amounts of solid SC-PEG or SS-PEG were added to a stirred solution of trypsin from bovine pancreas (100 mg, $\approx 4 \times 10^{-6}$ mol) in 20 mM $CaCl_2$ solution (20 ml) at pH 6. After all of the polymer went into solution ($\approx$2 min), the pH was raised to 8.5 for SC-PEG and 7.8 for SS-PEG and maintained at these respective levels for 30 min by automatic titration with sodium hydroxide (0.5 N). The solution was acidified to pH 3 with hydrochloric acid (0.1 N) and diafiltered against 1 mM HCl containing 20 mM $CaCl_2$ until no more free PEG was detected in the filtrate (27).

Similar procedures were used for the preparation of PEG-BSA conjugates. GF-HPLC analysis (Zorbax GF-450) showed good homogeneity of the PEG-protein conjugates and a substantial increase in molecular weight as compared with the parent proteins.

Determination of the number of PEG chains linked to a protein molecule. This parameter was calculated by multiplying the total number of amino groups on a particular protein (15 for trypsin, 59 for BSA) by the fraction of amines that were consumed by the modification, which was measured by fluorescamine assay (28) or, in the case of PEG-BSA conjugates, by TNBS assay (29).

Determination of Michaelis–Menten constants. Assays for amidolytic tryptic activity were performed at 37°C with a constant trypsin protein concentration of 1 μg/ml. N^{α}-Benzyloxycarbonyl-L-arginine *p*-nitroanilide was used as the substrate in concentrations varying from 0.02 to 1.71 mM in 50 mM Tris–HCl, pH 7.8, buffer that contained 10 mM $CaCl_2$. The constants were calculated from Lineweaver–Burk plots of the initial rates of the appearance of *p*-nitroaniline ($\epsilon_{410} = 8800\ \text{M}^{-1}\ \text{cm}^{-1}$).

Reactivity of activated PEGs as a function of pH. To triethanolamine-borate buffer (0.3 M, 1 ml) at the appropriate pH, a stock solution of NAL in water (50 mM, 0.1 ml) was added followed by a stock solution of the appropriate activated mPEG-5000 in CH_3CN (50 mM active acyl, 0.1 ml). The resultant solution was vortexed and incubated at 28°C for 1 h. A mixture of the same components but leaving out the acti-

vated PEG was used as a control. The TNBS assay according to Snyder and Sobocinski (30) was used to determine the unreacted NAL. The results of these measurements are shown in Fig. 4.

Preparation of PEG-acylated derivatives of tyrosine. The solution containing NATE (0.5 g, 20 mmol) in acetonitrile (3 ml) was treated with mPEG-chloroformate (6 g, 1.2 mmol) in methylene chloride (10 ml) followed by TEA (0.24 ml, 1.7 mmol). After 3 h toluene (20 ml) was added and the reaction flask was chilled to maximize TEA hydrochloride precipitation. The solution was filtered and evaporated to dryness. The residue was dissolved in warm ethyl acetate (50 ml), gravity filtered, and cooled to ≈20°C to facilitate precipitation of the product. Precipitation from ethyl acetate was repeated. The product (**3**) collected by filtration was dried *in vacuo*. The yield was 5.6 g (88%). I.R. (film on NaCl, cm^{-1}) characteristic bands at: 3391 (NH); 1761 (C=O, carbonate); 1741 (C=O, ester); 1679 (C=O, amide); 1114 (CH_2OCH_2).

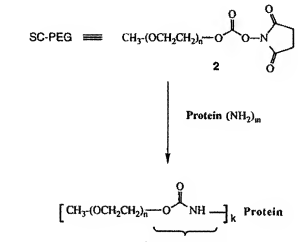
Compound **4** was obtained by a similar procedure using an equivalent amount of mPEG-succinate chloroanhydride (generated *in situ* with oxalyl chloride) instead of mPEG-chloroformate. I.R. (film on NaCl, cm^{-1}) characteristic bands at: 3391 (NH); 1758 (C=O, phenol ester); 1736 (C=O, ester); 1675 and 1663 (C=O, amide); 1508; 1114 (CH_2OCH_2).

TLC (ethyl acetate) analysis of compounds **3** and **4** on silica gel G plates showed an absence of free NATE (R_f = 0.49). The PEG-tyrosine compounds did not migrate under these conditions. The deacylation experiments described below provided further characterization of these compounds.

Deacylation of PEG-Tyr derivatives. Compound **3** in 0.1 M Tris hydrochloride, pH 7.5, containing hydroxylamine (0.5 M) showed characteristic tyrosine absorbance at λ_{max} = 275 nm within 10–15 min. Using $\Delta\epsilon_{278}$ = 1210 M^{-1} cm^{-1} (31) the content of tyrosine in this model compound was calculated as 1.67×10^{-4} mol/g (88% of the theoretical value of 1.895×10^{-4} mol/g). The same result was obtained when *N*-methylhydroxylamine was used instead of hydroxylamine.

Compound **4** hydrolyzed in 0.1 N NaOH showed characteristic tyrosine absorbance (at pH ≥ 12) with λ_{max} = 293 nm. Using ϵ_{293} = 2390 M^{-1} cm^{-1} (31) the calculated tyrosine content of **4** was 1.60×10^{-4} mol/g (85% of the theoretical value of 1.88×10^{-4} mol/g). When **4** was treated with hydroxylamine strong absorbance at λ_{max} = 260 nm was observed. Knowing the content of tyrosine in the model compound, calculations using ϵ_{260} = 8500 M^{-1} cm^{-1} for HOSu showed that 97% of the succinic acid diester (**4**) was converted to HOSu. Treatment of **4** with *N*-methylhydroxylamine (0.5 M) produced a characteristic tyrosine spectrum with λ_{max} = 275 nm. The content of tyrosine in **4** obtained in this experiment was 1.63×10^{-4} mol/g (87% of theoretical value), close to the value obtained from the alkaline hydrolysis experiment.

Treatment of PEG-modified proteins with deacylating agents. The appropriate deacylating agent (2 M, 0.3 ml) was added to the PEG-modified protein (≈1 mg/ml) in Tris hydrochloride, pH 7.5 (final volume 1.2 ml). The solution was mixed in the cuvette and the absorption spectrum scanned in the range 210–340 nm, looking for spectral changes around 270–280 nm, where tyrosine absorbs. The appropriate controls and blanks, without the deacylating agent and the modified protein, respectively, were prepared and scanned against the actual test samples. Neither one of the deacylating agents caused any absorption increments with SC-PEG-derived conju-



SCHEME 2. Use of SC-PEG for attachment of PEG to a protein.

gates. When PEG-trypsins obtained from SS-PEG were subjected to NH_2OH treatment, uv absorbance increments centering around 260 nm and not around 278 nm (the characteristic of deacylated tyrosyl residues) were observed. No spectral changes in the range of interest were observed when *N*-methylhydroxylamine was used.

RESULTS AND DISCUSSION

As seen in Scheme 2, the product of protein modification using SC-PEG has PEG chains grafted onto the polypeptide backbone through carbamate (urethane) linkages. The resistance of aliphatic urethanes to acidic, basic, and enzymatic hydrolysis is well documented (32, 33). In light of these precedents, it is reasonable to assume that the urethanes linking PEG chains to lysyl residues of proteins will not degrade under physiological conditions. The greater stability of the urethane linkage relative to the ester bond produced with use of SS-PEG (see Scheme 1) was expected to be the key difference between the two activated PEGs and the corresponding PEG-protein conjugates. Our studies indeed confirmed this expectation. Figure 1 shows the results of GF-HPLC measurements of free mPEG-5000 produced during the 37°C incubation of PEG-BSA conjugates derived from each of the activated PEGs. The higher stability of the SC-PEG-derived conjugate is very apparent.

Analogous to other protein acylating agents (21, 26, 34, 35), hydrolysis of the reagent itself is an important side reaction. In order to be useful as a protein modifying reagent, SC-PEG must react more rapidly with amino groups than itself undergoing hydrolysis in buffered aqueous media. A plot of first-order rate constants for hydrolysis and aminolysis versus pH (Fig. 2) demonstrates that this requirement is well satisfied by SC-PEG within a wide range that is useful for protein modification reactions. As pH increased the rate of aminolysis was found to rise more rapidly than the rate of hydrolysis. In these and in some of the subsequent experiments we used N^{α}-acetyllysine as a model compound mimicking the ϵ-amino group of a protein.

To estimate the relative reactivities of reagents **1** and **2**, we performed kinetic measurements of hydrolysis and aminolysis by NAL of both activated polymers in

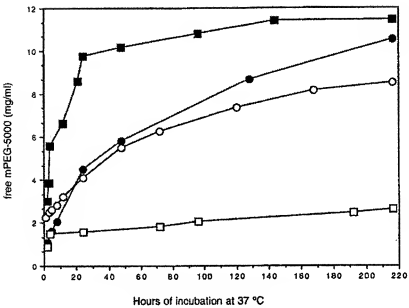


FIG. 1. Release of mPEG from PEG-BSA conjugates. Solutions of PEG-BSA conjugates (≈61% of amino groups modified), each at a concentration of 4 mg/ml, were incubated in the appropriate buffer. At given time intervals the concentration of mPEG-5000 was determined by GF-HPLC equipped with a refractive index detector. (●) SS-PEG conjugate, 0.1 M phosphate, pH 7.8; (○) SS-PEG conjugate, 50 mM PBS, pH 7.4; (■) SS-PEG conjugate, 0.1 M borate, pH 8.5; (□) SC-PEG conjugate, 0.1 M borate pH 8.5.

phosphate buffers, under conditions minimizing the hydrolysis of the aliphatic ester present in SS-PEG and its conjugates (Scheme 1). The results of these experiments are summarized in Table I. It is clear from these data that SS-PEG is somewhat more reactive among the two reagents. The difference in hydrolysis rates was larger than the difference in aminolysis rates; consequently, SC-PEG showed more favorable K_{am}/K_h ratios. Here too, the aminolysis reactions were accelerated by increasing pH and temperature to a greater extent than the corresponding hydrolysis. The slower hydrolysis of SC-PEG was also manifested in superior storage stability of the reagent (Fig. 3).

To find an optimal pH for protein modification reactions we reacted each activated PEG with an equimolar amount of NAL at different pHs, and measured the unreacted NAL using the TNBS assay (Fig. 4). The optimal pH for use of SC-PEG was found to be ca. 9.3. It is not advisable to use SS-PEG at pH > 8.0, due to the limited stability of PEG-succinate ester. However, the higher reactivity of this reagent compensates for this limitation and it was found to be very reactive even at pH values below 8.0.

Modifications of Trypsin

Further evaluation of the activated PEG derivatives **1** and **2** was obtained through results from a series of modifications on trypsin. Trypsin was chosen as a model protein for this work for the following reasons: (a) it is inexpensive and readily available in high purity, and (b) properties of this enzyme and many of its chemically modified derivatives are well documented in the literature.

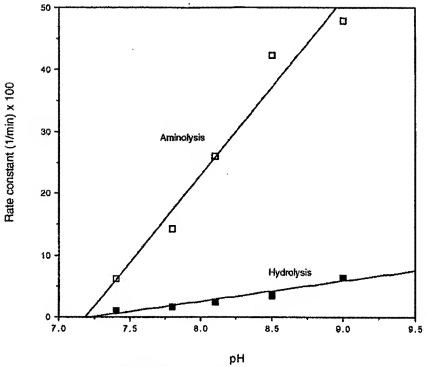


FIG. 2. Hydrolysis and aminolysis of SC-PEG at 27°C as a function of pH.

A number of modifications were carried out using different molar ratios of 1 or 2 to trypsin at pH values in the range of 7.5–8.5, for 30 min. The products were purified by extensive diafiltration[3] and the degrees of modification were determined by fluorometric assay, according to Stocks *et al.* (28). All PEG-modified trypsin derivatives were essentially lacking (<1% of native) proteolytic activity as determined by the Azocoll assay (36). The lack of proteolytic activity of PEG-trypsins is in complete agreement with the earlier work of Abuchowski and Davis on the modification of trypsin with cyanuric chloride-activated PEG (37).

The specific activities of representative SS- and SC-PEG-modified trypsins toward low-molecular-weight substrates are summarized in Table II. Esterolytic activity of the modified enzymes, as assayed by using N^{α}-benzoyl-L-arginine ethyl ester (BAEE), was essentially unaffected by the modifications. All modified enzymes ranged from 92 to 112% of the activity found for the native enzyme.

Amidolytic activities toward N^{α}-benzoyl-DL-arginine *p*-nitroanilide (BAPA) and N^{α}-benzyloxycarbonyl-L-arginine *p*-nitroanilide (ZAPA) were tested. An increase in the ability of modified enzymes to catalyze the hydrolysis of *para*-nitroanilides was consistently observed (Table II). Michaelis–Menten kinetic constants for several SC- and SS-PEG-modified trypsins were measured using ZAPA as a substrate. These

[3] Omission or incompletion of the diafiltration step, particularly when 2 was used as a protein modifier, resulted in erroneous TNBS and/or fluorescamine assay results. The mechanism responsible for this phenomenon was elucidated and will be described elsewhere.

TABLE I

Comparison of First-Order Rate Constants for Hydrolysis (K_h) and Aminolysis (K_{am}) of SC-PEG and SS-PEG

pH	Temperature (°C)	Hydrolysis: $K_h(min^{-1}) \times 10^3$ and $[t_{1/2}(min)]$ SC-PEG	SS-PEG	Aminolysis: $K_{am}(min^{-1}) \times 10^3$ and $[K_{am}/K_h]$ SC-PEG	SS-PEG
7.0	4	0.87 [793]	1.84 [376]	2.64 [3.0]	3.74 [2.0]
	27	6.05 [115]	10.4 [67]	26.4 [4.4]	41.4 [4.0]
	37	14.2 [49]	25.9 [27]	81.7 [5.8]	104 [4.0]
7.4	22	5.37 [129]	10.7 [65]	29.1 [5.4]	42.7 [4.0]
	27	9.0 [77]	16.0 [43]	48.6 [5.4]	73.6 [4.6]
	37	19.3 [36]	37.6 [18]	145 [7.5]	193 [5.1]
7.8	4	1.37 [505]	2.58 [268]	12.4 [9.1]	15.0 [5.8]
	27	10.3 [67]	21.6 [32]	130 [12.6]	152 [7.0]
	37	21.8 [32]	48.8 [14]	226 [10.6]	267 [5.5]

results (summarized in Table III) show that while V_{max}, k_{cat}, and k_{cat}/K_m were increasing gradually with the extent of modifications, K_m values were decreasing. This indicates that the attachment of PEG caused an increase in both the rate of turnover of ZAPA and its affinity toward the modified enzymes.

According to several reports (37–41), enhanced activities toward simple substrates were observed as a consequence of chemical modification of trypsin. These increases in esterolytic (39, 40) and amidolytic (40, 41) activities were attributed by some authors to modification of tyrosine residues. On the other hand, Kanazawa *et al.* (38) described a trypsin modified exclusively at its lysyl residues which had 2.5-fold increased activity toward BAPA; and Riordan *et al.* (42) claimed that up to 6.7 of the 10 tyrosyls of trypsin can be acetylated without change in enzymatic activity.

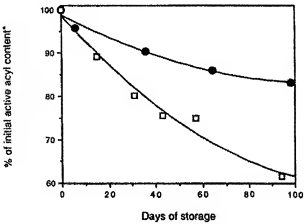


FIG. 3. Stability of SC-PEG (●) and SS-PEG (□) at 22°C. The activated PEGs were stored in the form of a fine powder in tightly closed polypropylene containers. At given time intervals samples of each polymer were titrated for active acyl content according to Kalir *et al.* (25). *The initial active acyl contents of both activated PEGs were 1.91×10^{-4} mol/g.

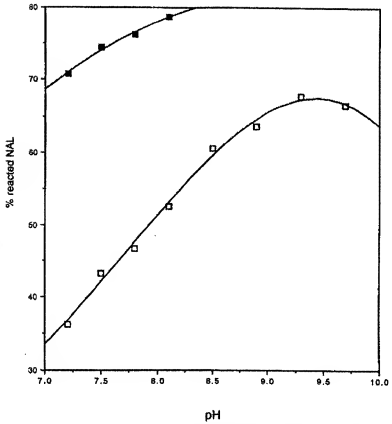


FIG. 4. Reactivity of SC-PEG (□) and SS-PEG (■) as a function of pH.

Studies of Possible Acylation of Tyrosyl Residues

These reports prompted our interest in analyzing PEG-trypsin derivatives for the presence of acylated tyrosyl residues. It is well known that phenol esters, e.g., acetates, formed upon modification of tyrosyl residues are readily deacylated by treatment with hydroxylamine. This process causes a measurable increase in the uv absorbance ($\Delta\epsilon_{278} = 1210\ M^{-1} \cdot cm^{-1}$), thus allowing the quantitation of the number of modified tyrosine moieties (31, 43). In order to use a similar approach on SC- and SS-PEG-protein conjugates, the lability of the relevant tyrosine carbonate and ester linkages toward hydroxylamine had to be established. With this goal in mind we prepared model compounds **3** and **4** by reacting N^{α}-acetyltyrosine ethyl ester with mPEG-chloroformate and mPEG-succinoyl chloride, respectively. Treatment of **3** with hydroxylamine readily regenerated free phenol groups of tyrosine as was evidenced by the appearance of tyrosine-characteristic uv spectra (Scheme 3). Analysis of the reaction mixture of **4** with hydroxylamine showed no characteristic tyrosine absorbance. Instead, the spectrum showed an intense absorbance at $\lambda_{max} = 260$ nm, due to the formation of HOSu from the succinic acid residue of **4** and one equivalent of NH_2OH. We reasoned that the use of *N*-methylhydroxylamine instead of hydroxylamine in the deacylation of **4** would eliminate the possibility of succinimide ring formation (see Scheme 4). Indeed, a typical tyrosine spectrum was obtained from the

TABLE II

Summary of Modifications, Esterolytic and Amidolytic activities of Trypsin Derivatives

Trypsin derivatives[a]	*Modification (%)*[b]	*BAEE*[c] *(u/mg)*	*% of native*	*BAPA*[d] *(u/mg)*	*% of native*	*ZAPA*[d] *(u/mg)*	*% of native*
Native trypsin	0	92.4	100	1.26	100	7.81	100
SC-PEG$_n$-trypsin							
$n = 6$	42.3	103	112	2.26	179	15.3	196
$n = 7$	45.8	87.9	95.1	2.38	188	17.5	224
$n = 9$	58.8	90.1	97.5	2.67	212	18.9	242
$n = 12$	77.9	85.1	92.2	3.83	304	25.5	326
SS-PEG$_n$-trypsin							
$n = 7$	44.8	102	110	3.25	258	18.8	241
$n = 12$	77.0	94.3	102	4.34	344	24.7	316

[a] For SX-PEG$_n$-trypsin, $n = 15 \times$ (% modification)/100 and rounded to the nearest integer.

[b] The percentage of amino groups modified was determined by the fluorescamine assay (28).

[c] The assay was performed at pH 7.8, 37°C, with a BAEE concentration of 0.5 mM. $\epsilon_{253} = 808\ M^{-1}\ cm^{-1}$ was used for calculations.

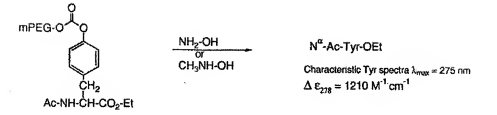
[d] The amidolytic assays were performed in 50 mM Tris, pH 8.1, 10 mM $CaCl_2$, at 37°C, with a substrate concentration of 1 mM. $\epsilon_{410} = 8800\ M^{-1}\ cm^{-1}$ was used for calculations.

uv scan of this reaction solution. *N*-Methylhydroxylamine proved to be an equally effective deacylating reagent as hydroxylamine for mPEG-carbonate of NATE (3). These experiments established that, if tyrosyl residues of a protein were acylated by SC- or SS-PEG, they could be cleaved by a suitable nucleophilic agent and detected by uv absorbance. They also showed that while both SS-PEG- and SC-PEG-modified proteins can be checked for phenol group acylation using *N*-methylhydroxylamine as the deacylating agent, only the latter type of PEG-proteins can be analyzed using hydroxylamine. When trypsin conjugates derived from each one of the activated PEGs were exposed to such deacylating reagents, no absorbance increments in the range 270–285 nm were observed, indicating that neither one of the activated polymers reacted with tyrosyl residues of the trypsin molecule.

TABLE III

Michaelis–Menten Constants for the Amidolytic Activity of Native Trypsin and Its mPEG Derivatives

Trypsin derivatives	K_m *(mM)*	V_{max} *(μM/min)*	k_{cat} *(min^{-1})*	k_{cat}/K_m *($mM^{-1} \cdot min^{-1}$)*
Native trypsin	1.08	15.7	378	349
SC-PEG$_n$-trypsin				
$n = 7$	0.29	19.6	470	1626
$n = 9$	0.21	20.2	484	2290
$n = 12$	0.11	22.9	549	4973
SS-PEG$_n$-trypsin				
$n = 7$	0.21	18.6	447	2172
$n = 12$	0.13	22.5	539	4159



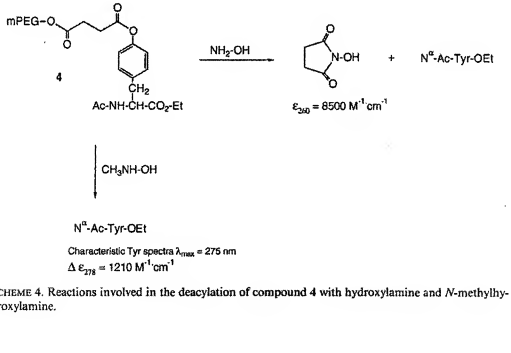
3

SCHEME 3. Deacylation of mPEG carbonate of NATE (3) with hydroxylamine or *N*-methylhydroxylamine.

In a different set of experiments, using N^{α}-acetyl derivatives of lysine and tyrosine (NAL and NAT), we measured the extent of the tyrosine interference in the reaction between amine and each one of the activated polymers. The results of these experiments (Table IV) demonstrated that the presence of phenol groups of NAT in the reaction between NAL and each one of the activated PEGs had little or no influence on the amount of reacted amine. Only when the NAT/NAL ratio was 2.5-fold and higher was some decrease in the amount of NAL that reacted with SS-PEG observed. In the case of SC-PEG, a 5-fold excess of NAT over NAL was needed to cause a perceivable decrease in the extent of the amine reaction. Such ratios of phenol to amino groups are unrealistic for proteins. The fact that under such exaggerated conditions NAT had very little ability to compete with NAL makes the likelihood of acylation of tyrosyl residues of proteins with either one of the activated polymers extremely low.

CONCLUSIONS

Compared to the conventionally used SS-PEG, the new reagent, SC-PEG, is slightly less reactive yet more selective. This is evidenced by its higher K_{am}/K_h ratios,



4

SCHEME 4. Reactions involved in the deacylation of compound 4 with hydroxylamine and *N*-methylhydroxylamine.

TABLE IV

Effect of NAT on the Extent of Reaction of SS-PEG and SC-PEG with NAL[a]

	% of NAL reacted with	
NAT/NAL	*SS-PEG*	*SC-PEG*
0	78.6 (100)[b]	53.8 (100)
1	77.2 (98.2)	55.3 (103)
2.5	74.5 (94.8)	52.6 (97.8)
5.0	68.5 (87.2)	48.6 (90.3)

[a] Reactions were performed at pH 8, with the specified ratios of NAT/NAL, using the procedures described under Experimental, "Reactivity of activated PEGs as a function of pH."

[b] The numbers in parentheses represent the values of the percentage of NAL reaction divided by the percentage of NAL reaction in the absence of NAT (when NAT/NAL = 0).

better storage stability, and lower interference of NAT in the SC-PEG reaction with amine. Protein modification reactions with the new reagent can be performed in a broad pH range, with optimal reactivity at pH $\approx$ 9.3. The urethane-linked PEG-protein conjugates obtained through the use of SC-PEG are chemically more stable than SS-PEG-modified proteins. This is the single most important difference between the PEG-conjugates derived from the two reagents, and may be significant depending upon the application.

Both SS- and SC-PEG showed a high reactivity toward trypsin, yielding conjugates with comparable degrees of modification and similar properties: lack of proteolytic activity, preservation of esterolytic activity, and dramatically increased activity toward *p*-nitroanilide substrates.

Our results indicate that the observed enhanced activity of PEG-modified trypsins toward nitroanilide substrates is independent of the modification of tyrosyl residues. At the present time we cannot offer a plausible explanation as to the cause of this activity enhancement. In light of existing literature precedents, it is unlikely that significant conformational changes occurred as a result of PEG attachment to proteins (44, 45). Possibly, the increased amphipatic nature of the modified enzymes and preferential partitioning of the hydrophobic nitroanilide substrates into a PEG-rich environment of the modified enzymes are the reasons for the observed phenomena. Several cases of enhanced activity obtained as a result of PEG attachment to enzymes have been described in the literature: Beauchamp *et al.* (13) observed an increase in α_2-macroglobulin-trypsin amidolytic activity as a result of PEG attachment to a preformed complex of these proteins. An example of enhanced activity of PEG-β-galactosidase toward hydrophobic substrates and substantial activity toward GM_1-ganglioside, which cannot be hydrolyzed by the native enzyme in the absence of detergents, was reported (46).

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Technical Report

Development of a Rapid Method for the PEGylation of Adenoviruses with Enhanced Transduction and Improved Stability under Harsh Storage Conditions

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ABSTRACT

PEGylation is the covalent attachment of activated monomethoxy poly(ethylene) glycols (MPEGs) to free lysine groups of therapeutic proteins. This technology has enhanced the physical stability of proteins and ablated humoral immune responses generated against them. In this study, adenoviral vectors were modified with MPEGs activated by cyanuric chloride, succinimidyl succinate, and tresyl chloride. Under proper buffering conditions, reactions were complete within 2 hr. Transduction efficiency of PEGylated adenoviruses was not compromised by neutralizing antibodies to native adenovirus *in vitro*. These preparations retained titers that were significantly greater than those of the unconjugated virus after storage at 42, 25, 4, and −20°C. Stability profiles of PEGylated preparations at −20°C suggest that glycerol could be eliminated from formulations without significant loss of viral titer. PEGylated adenoviruses produced a two- to threefold increase in transduction in the lung when administered by intratracheal injection and a fivefold increase in transduction in the liver when administered intravenously.

OVERALL SUMMARY

Current storage conditions (10% glycerol, −80°C) of adenoviral vectors limit the ability of the virus to be shipped to remote sites and require dilution prior to administration in the clinic. In this report, we extend the physical stability of adenoviral vectors by covalent attachment of various polyethylene glycols (PEGs) to viral capsid proteins. PEGylated preparations were stored in potassium phosphate-buffered saline (pH 7.4) at −20, 4, 25, and 42°C for 6 months. Lac-forming assays were used to titer all preparations. PEGylated virus retained titers that were significantly greater than that of unmodified virus under each study condition. Addition of glycerol to PEGylated preparations did not enhance viral stability at −20°C, indicating that it could be eliminated from formulations. TMPEG and SSPEG preparations showed negligible loss of titer at room temperature for 24 hr, which would allow for priority shipment of vector in the absence of ice.

INTRODUCTION

Recombinant adenoviral (Ad) vectors have commanded considerable attention as gene delivery vehicles since high-titer replication-deficie nt recombinant viral preparations can be easily generated (Yeh and Perricaudet, 1997), the viruses can be engineered to accommodate large DNA inserts (Graham and Prevec, 1995), and they are capable of producing large amounts of gene products in a wide variety of dividing and nondividing cells (Hitt *et al.*, 1997). Enthusiasm for extensive use of adenoviral vectors, however, has diminished because of cellular immune responses generated against transduced cells expressing viral and transgene-derived proteins, which limit the length of gene expression (Yang *et al.*, 1996a,b). Furthermore, transduction efficiency in the lung for diseases such as cystic fibrosis and the liver for diseases caused by genetic protein deficiencies such as hemophilia (Connelly *et al.*, 1998) and urea cycle disorders (Zimmer *et al.*, 1999) is severely hampered at the time of vector readministration because of neutralizing antibodies

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generated against the viral proteins (Kozarsky *et al.*, 1994; Yang *et al.*, 1995; Kaplan *et al.*, 1996). This vector is also hampered by poor physical stability at ambient temperatures (Croyle *et al.*, 1998). Current storage conditions (10% glycerol, phosphate-buffered saline, pH 7.4, −80°C) can maintain an adequate titer of adenoviral stocks, but limit the ability of the vectors to be shipped to remote sites and requires dilution prior to administration in the clinic.

Covalent modification of proteins and enzymes with functionalized polyethylene glycol (PEG) has been extensively studied since the late 1970s (Delgado *et al.*, 1992). PEG is an uncharged, hydrophilic, linear polymer that is nonimmunogenic and has a low order of toxicity (Montaguti *et al.*, 1994; Hjortkjaer *et al.*, 1999). PEG is approved by the Food and Drug Administration (FDA) for use in drugs (parenterals, topicals, suppositories, nasal sprays), foods, and cosmetics. More than 40 therapeutic proteins have been modified by PEG. Currently, five PEG-modified proteins are used in the clinic: PEG–L-asparaginase (Asselin, 1999), PEG–superoxide dismutase (Corvo *et al.*, 1999), PEG–adenosine deaminase (Hershfield, 1995), PEG–interleukin 2 (Kelleher, *et al.* 1998), and PEG–α interferon (Nieforth *et al.*, 1996). In most cases, these proteins have shown improved therapeutic efficacy with enhanced circulation half-life *in vivo*, reduced immunogenicity, enhanced solubility, and suitable *in vivo* bioactivity.

O'Riordan *et al.* have developed a process to covalently link various polyethylene glycols to the capsid proteins of adenovirus (O'Riordan *et al.*, 1999). The present article describes three conjugation methods with shortened reaction times that sufficiently modify the viral capsid and the physical stability of the adenovirus under extreme storage conditions. Transduction efficiency of these new vectors when administered by the intratracheal and intravenous routes was also assessed.

MATERIALS AND METHODS

Production of conjugated adenoviral vectors

First-generation adenoviral vectors expressing β-galactosidase (H5010.CMV.*lacZ*) were used for these studies and were amplified in 293 cells by a modification of established methods and purified from cell lysates by banding twice on CsCl gradients (Graham and Van der, 1973). Aliquots of virus were desalted on Econo-Pac 10DG disposable chromatography columns (Bio-Rad, Hercules, CA) and equilibrated with the respective buffer for optimal conjugation (see below). Viral concentrations were determined by UV spectrophotometric analysis at 260 nm. Transduction titer (i.e., LacZ-forming units or LFU) was determined by limiting dilution infections of 293 cells. The particle-to-LacZ-forming unit ratio of both conjugated and unconjugated virus was approximately 100. Protein content of Ad preparations was determined by a microplate assay with Bio-Rad DC Protein assay reagents and bovine serum albumin as a standard.

Three types of activated monomethoxy polyethylene glycol (MPEG) were used in this study: tresyl-MPEG (TMPEG), succinimidyl succinate MPEG (SSPEG), and cyanuric chloride MPEG (CCPEG). All three were obtained from Sigma (St. Louis, MO). Conjugation reactions were performed by a modification of established methods (Jackson *et al.*, 1987; Delgado *et al.*, 1990; Kita *et al.*, 1990). For TMPEG, adenoviral bands were desalted into 10 m*M* potassium phosphate buffer, pH 7.4. Virus was desalted into 0.2 *M* sodium phosphate (pH 7.2) and 0.1 *M* sodium tetraborate (pH 9.2) buffers for conjugation with SSPEG and CCPEG, respectively. A 10:1 PEG-to-virus ratio (amount of PEG:amount of Ad protein) provided the most efficient reaction times and produced minimal loss of infectivity of the virus. All conjugation reactions were performed at 25°C with gentle stirring. Reactions were stopped by addition of 10× L-lysine. Unreacted PEG, excess lysine, and reaction by-products were eliminated by buffer exchange over a Sephadex G-50 column equilibrated with 10 m*M* potassium-buffered saline (KPBS), pH 7.4. Fractions containing virus were identified by UV spectrophotometric analysis at 260 nm and pooled for further study.

Fluorescamine assay

A standard fluorescamine assay was used to estimate the degree of modification of adenovirus capsids by activated polyethylene glycols, using a modification of established methods (Stocks *et al.*, 1986). Serial dilutions were made from each sample in a volume of 1.5 ml of 10 m*M* sodium phosphate buffer, pH 7.4. Fluorescamine (0.3 mg/ml; Sigma) in acetone (0.5 ml) was added to each dilution while vortexing. Fluorescence was measured on a spectrofluorimeter (Photon Technology International, Monmouth Junction, NJ) (excitation, 390 nm; emission, 475 nm). The resulting fluorescence is proportional to the concentration of free amino groups on the virus capsid. Standard curves were generated for each time point by plotting protein concentration versus fluorescence units. Degree of modification was obtained as the ratio between the slopes of conjugated and unconjugated viruses at similar time points.

Partitioning assays

Partition coefficients of native and PEGylated viruses were determined as described previously (Delgado *et al.*, 1994). Partitioning assays were performed at 25°C in single microcentrifuge tubes containing 1 g of a two-phase system of 4.75% (w/w) PEG 8000 (Sigma), 4.75% Dextran T500 (Amersham Pharmacia Biotech, Piscataway, NJ), in 0.15 *M* NaCl-containing 0.01 *M* sodium phosphate buffer, pH 6.8. Adenovirus and PEGylated adenovirus were incorporated into the system by replacing 0.1 g of the water used to prepare the phases with 0.1 g of virus in coupling buffer. Samples were mixed 30–40 times by inversion and left to settle under gravity until complete separation of the phases was achieved. Aliquots from the top and bottom phases were analyzed for protein concentration with Bio-Rad DC protein assay reagents and bovine serum albumin as a standard. The partition coefficient (K) is determined by the ratio between the protein concentrations in the top and bottom phases.

Zeta potential

The zeta potential of PEGylated adenovirus preparations in 10 m*M* potassium phosphate buffer, pH 7.4, was determined by laser Doppler anemometry (Zetasizer 3000; Malvern Instruments, Southboro, MA) in a thermostatted microelectrophoresis unit.

Neturalization assays

Conjugated adenovirus preparations were incubated with immune or nonimmune plasma from C57BL/6 mice for 30 min with gentle shaking. Immune serum was derived from C57BL/6 mice bled 28 days after intravenous injection of Ad vector. Two hundred-microliter aliquots (equivalent to a multiplicity of infection of 50) were applied to cells in triplicate. After a 2-hr incubation, vector was replaced with complete medium. Twenty-four hours after infection, cells were washed with PBS, fixed in 0.5% glutaraldehyde, and assessed for β-galactosidase expression histochemically.

Administration of PEGylated vectors to immunocompetent animals

C57BL/6 (H-2^b) mice (6–8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). Preparations were administered either via the tail vein (1×10^{11} particles in 100 μl of KPBS) or intratracheally (5×10^{10} particles in 50 μl of KPBS). Animals were necropsied 4 days later and excised tissues were washed twice in cold PBS and stored in cold Dulbecco's modified Eagle's medium (DMEM) for processing. Tissues were homogenized in 1 ml of lysis buffer, using a Brinkmann (Westbury, NY) Polytron. Extracts were centrifuged at 14,000 rpm for 10 min. Protein concentration of supernatants was determined with Bio-Rad DC protein assay reagents and bovine serum albumin as a standard. Extracts were quick-frozen in dry ice and stored at $-80°C$ until assayed. β-Galactosidase (β-Gal) concentrations were determined with a β-Gal enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer instructions.

RESULTS

Conjugation of activated MPEGs to adenoviral capsids occurs rapidly with minimal loss of viral infectivity

Conjugation of PEG to proteins generally results in some loss of bioactivity. Because adenoviral infection relies on the interaction of several protein–receptor interactions, PEGylation could compromise the ability of the virus to transduce target tissues (Bergelson *et al.*, 1997; Nemerow and Stewart, 1999). The TMPEG preparation retained viral titer over the entire reaction period whereas the SSPEG preparation lost 70% of the original titer when the conjugation reaction was complete (Fig. 1A). Premature termination of the SSPEG reaction (i.e.,

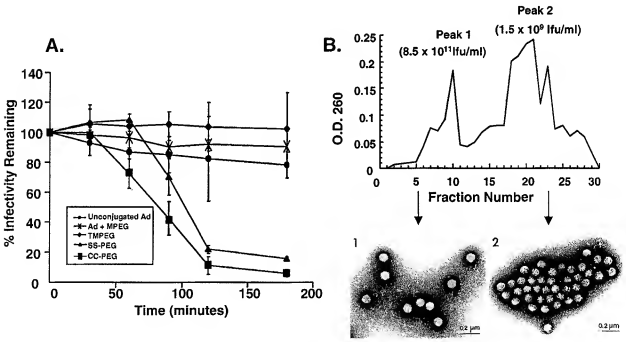

FIG. 1. Effect of PEGylation on transduction efficiency of adenoviral preparations. (A) Samples of virus were taken at various times over a 3-hr reaction period, serially diluted in DMEM supplemented with 2% FBS, and added to confluent 293 cells. Unconjugated virus was subjected to gentle stirring at room temperature, as were the conjugated viruses. The TMPEG preparation displayed no significant loss of infectivity during the conjugation reaction. Adenovirus sham treated with unactivated MPEG (Ad + MPEG) in 10 mM potassium phosphate buffer also displayed a minimal loss of infectivity over the reaction period. Data reported are the result of three separate experiments. Error bars represent the standard deviation of the data. (B) Significant loss of transduction efficiency in preparations modified by CCPEG is due to severe cross-linking of virions by the polymer. During the final buffer exchange over a Sephadex G-50 column, the CCPEG preparations produced two distinct viral populations: one that, despite its estimated particle count, had an extremely low transduction efficiency due to the presence of large viral aggregates in the preparation (peak 2); the other population, a suspension of single virus particles, had high transduction efficiency with respect to its estimated concentration (peak 1). Only the virus in the first peak was isolated and employed in additional studies.

at 75 min, when it was 70% complete) resulted in a preparation with 90% residual activity. Modification of the virus with CCPEG was complete in 90 min; however, only 11% of the original infectivity remained. Fractionation of this preparation over a Sephadex G-50 column produced distinct viral populations (Fig. 1B). The first peak represented PEGylated monomer virus with high infectivity whereas the second peak contained aggregated virus (per electron microscopy and static laser light scatter, data not shown) with low activity. Only the first peak was isolated and used in additional studies. Addition of unactivated MPEG to Ad preparations had no effect on infectivity as compared with vector in buffer alone.

Characterization of Ad–PEG complexes

Several physical tests were performed to confirm that activated PEG molecules were successfully conjugated to the adenovirus capsid. Partitioning of the viral preparations in an aqueous two-phase system and calculation of partition coefficients (K) demonstrated that the viral capsid was significantly modified. K values shifted from 0.7 for unlabeled virus to 1.76 and 1.96 for TMPEG and SSPEG preparations, respectively. The CCPEG preparation demonstrated the highest level of conjugation, with a K value of 3.56.

Zeta potential analysis revealed that surface charge of the adenovirus capsid significantly changed from −48.1 mV to −27.8 and −24.2 mV when conjugated to TMPEG and SSPEG, respectively. The CCPEG preparation demonstrated the greatest shift toward neutrality, to −16.2 mV.

Adenovirus–PEG complexes are protected from neutralization by immune serum

PEGylated preparations were added to HeLa cells in the presence of neutralizing antibodies to adenovirus capsid proteins and transduction levels were compared with that of the same preparation incubated in nonimmune serum. Transduction efficiency of the unconjugated virus was significantly reduced by neutralizing antibodies (Fig. 2). The TMPEG preparation did shield the virus partially from neutralization, whereas transduction of the SSPEG and CCPEG preparations was unaffected by the presence of neturalizing antibody.

PEGylated adenoviruses are significantly more stable under various storage conditions than unconjugated virus

We assessed the ability of PEGylation to stabilize virus at a wide variety of temperatures (Fig. 3). Preparations were stored in potassium phosphate buffered saline (KPBS) with or without the addition of 10% glycerol at −20°C and at 4°C. Figure 3A presents the stability data at 4°C. Unconjugated adenovirus suffered a drop in titer of 1 log after storage at 4°C for 8 hr, falling to undetectable levels 7 days after storage. This rapid degradation of viral capsids is readily detected by electron microscopy. After 24 hr at 4°C, the unconjugated viral preparation consists mainly of single viral capsid proteins, mostly hexons (Fig. 4B, arrowheads); only a few intact viral particles could be detected (Fig. 4C, arrows). The CCPEG preparation was the

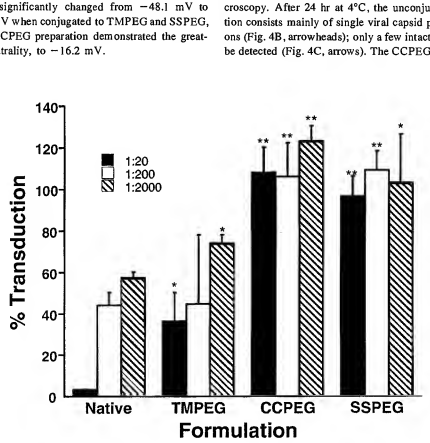

FIG. 2. Attachment of MPEGs to adenovirus capsids allows for efficient transduction in the presence of neutralizing antibody *in vitro*. Adenovirus at an MOI of 50 was incubated with immune serum from C57B/6 mice for 30 min at room temperature and added to subconfluent HeLa cells. Transduction levels are reported as the ratio of positive cells from virus incubated with immune serum to the number of cells transduced by virus in the presence of nonimmune serum. Data are the result of triplicate samples from two separate experiments. $^{**}p < 0.001$, $^{*}p < 0.05$ (Student t test).

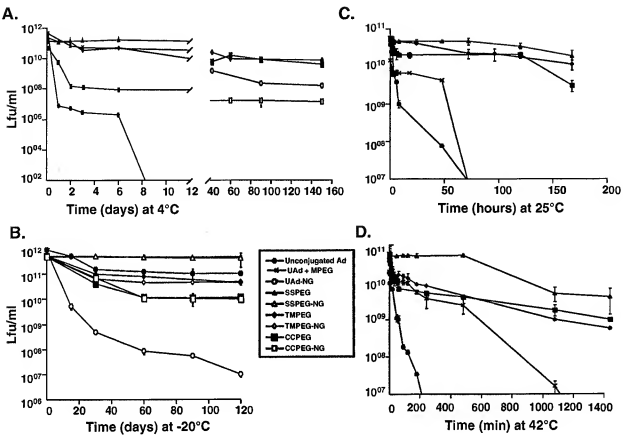

FIG. 3. Effect of PEGylation on the physical stability of adenovirus at (A) 4°C, (B) −20°C, (C) 25°C, and (D) 42°C. One-milliliter aliquots of PEGylated adenovirus preparations (H5010CMV*LacZ*) in 10 m*M* potassium phosphate buffer (pH 7.4) were stored in stoppered, sealed, borosilicate glass vials at 4, 25, 42, and −20°C. Samples of virus were taken at various times, serially diluted in DMEM supplemented with 2% FBS, and added to subconfluent 293 cells. Data are the result of determination of β-galactosidase-exp ressing cells from four samples of a single lot of PEGylated virus. Error bars represent the standard deviation of the data. NG, no glycerol.

least stable of the remaining preparations, as titer fell 1 log after incubation at 4°C for 24 hr. Electron micrographs display unequivocal evidence that this preparation is significantly more stable than unconjugated virus, as photos taken 12 days after storage reveal intact virions (Fig. 4D). TMPEG preparations demonstrated a negligible loss in titer at 4°C for 1 week; after 42 days, the titer fell by 2 logs and remained at this level for the duration of the study. At this time, the preparation mainly consisted of intact viral capsids with only a few compromised virions (Fig. 4E, arrow). The SSPEG preparation underwent an initial drop in titer of 1 log after 8 hr at 4°C and maintained titer for up to 150 days. This preparation was indistinguishable from freshly purified virions on inspection by electron microscopy 120 days after storage at 4°C (Fig. 4F).

Stability studies of PEGylated preparations at −20°C revealed that the addition of glycerol was not necessary for maintenance of titer (Fig. 3B). In the absence of glycerol, titers of unconjugated virions dropped 5.5 logs after 15 days at −20°C in KPBS. Degradation of the virus steadily continued at the rate of 1 log/month for the remainder of the study. Addition of 10% glycerol significantly enhanced stability as only 1 log unit of titer was lost in the unconjugated preparations stored at −20°C over 4 months. SSPEG-conjugated virions were the most stable of all the preparations studied, with a drop in titer of 0.8 log over 120 days at −20°C; addition of glycerol only slightly enhanced stability. The titer of the TMPEG preparation was the most sensitive to the addition of glycerol. The preparation without glycerol demonstrated a drop of 1 log after 90 days in storage, whereas the titer of the preparation that contained glycerol dropped only 0.5 log unit. CCPEG virions were least stable, with a 4-log decrease in infectious virus after 90 days of storage at −20°C; glycerol did not help.

PEGylation enhances adenoviral stability under extreme storage conditions

Even though the stability data for PEGylated viral preparations at 4 and −20°C were encouraging, these conditions would still require shipment on ice to various clinical sites. To assess the possibility of vector shipment under ambient conditions, samples were stored in KPBS at 25°C (Fig. 3C) and at 42°C (Fig. 3D). On storage at 25°C, unconjugated adenovirus

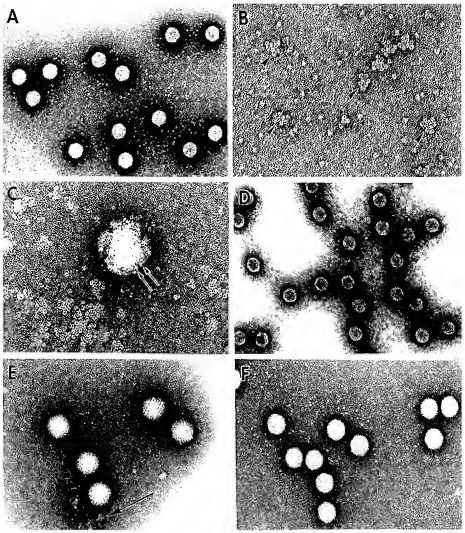

FIG. 4. PEGylation of adenovirus maintains integrity of viral structure after extensive storage in potassium phosphate buffer at 4°C. (**A**) Electron micrograph of freshly purified adenovirus virions. (**B**) Electron micrograph of the same preparation after 24-hr storage at 4°C. Most samples contained only viral capsid proteins such as hexons (arrowheads), pentons, and fibers. (**C**) On further inspection, a few intact virions could be detected. (**D**) Electron micrograph of adenovirus conjugated with CCPEG after storage for 12 days at 4°C. (**E**) Electron micrograph of virions conjugated with TMPEG after 42 days at 4°C. (**F**) Electron micrograph of viral particles stored in the presence of unactivated SSPEG after storage for 4 months at 4°C. This preparation was more stable than other PEGylated viruses at 4°C.

dropped in titer at a rate of 1 log/day (Fig. 3C). Addition of monomethoxy polyethylene glycol to the adenovirus preparation slightly enhanced stability of unconjugated adenoviral preparations, with the loss of titer at an average rate of 0.6 log unit/day.

CCPEG preparations were stable at room temperature. An initial drop in titer from 5.84×10^{10} to 3.18×10^{10} LFU/ml was detected after 6 hr, which subsequently stabilized for 1 week. TMPEG preparations were stable for 24 hr while the titer of the SSPEG preparation remained constant for up to 5 days.

When stored at 42°C, titer of unconjugated adenovirus preparations initially fell at a steady rate of approximately 1 log every 45 min. Addition of inactivated MPEG delayed the loss, which did eventually diminish 2 logs over 3 hr. TMPEG and CCPEG preparations demonstrated similar degradation rates with titers falling 1.5 log unit over the study period. The SSPEG preparation was the most stable with negligible loss of titer for 18 hr at this condition.

PEGylation enhances adenovirus-mediated gene transduction on administration to the liver and lung of naive immunocompetent animals

Preparations were assessed for transduction efficiency and administered by either intratracheal or intravenous injection at doses equivalent to 5×10^{10} particles and 1×10^{11} particles, respectively. When administered to the lung, unconjugated virus produced $1.42 \times 10^4 \pm 3.6 \times 10^3$ pg of β-galactosidase/mg protein (Fig. 5A). The CCPEG and SSPEG preparations produced β-galactosidase expression levels twofold

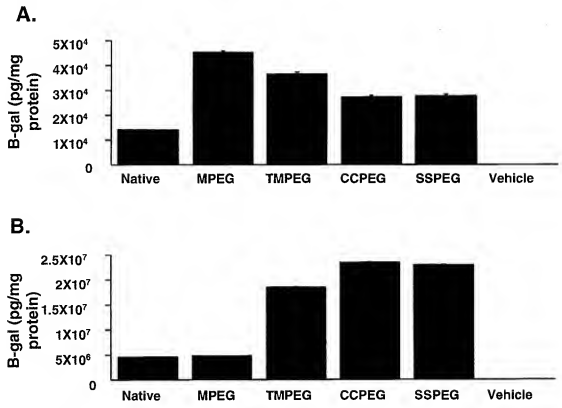

FIG. 5. PEGylated adenovirus enhances the transduction efficiency in (A) lung and (B) liver. C57BL/6 mice were injected either intratracheally (5×10^{10} particles/ml) or intravenously (1×10^{11} particles/ml) with either unconjugated or PEGylated Ad encoded with the *lacZ* gene. Four days after injection, animals were necropsied and gene expression assessed in the target organ by a β-galactosidase ELISA. Data reflect the average values from 10 animals in two separate experiments. Error bars represent the standard error of the mean of the data.

higher than unconjugated virus. The TMPEG preparation demonstrated the largest increase in transduction with a 2.5-fold increase in β-galactosidase. Addition of inactivated MPEG to Ad preparations raised transduction to a level three times that of the unconjugated virus.

Administration of unconjugated adenovirus intravenously yielded β-galactosidase levels of $3.0 \times 10^6 \pm 1.1 \times 10^5$ pg/mg of protein in the liver (Fig. 5B). *In vivo* transduction of liver was sixfold higher with TMPEG, CCPEG, and SSPEG. Addition of MPEG to the viral preparation slightly raised transduction levels beyond that of the unconjugated virus.

DISCUSSION

Established protocols for the PEGylation of proteins with various activated forms of methoxypolyethylene glycols (MPEGs) generally reach completion within 1–2 hr at room temperature under appropriate buffer conditions (Inada *et al.*, 1994). A method for the PEGylation of adenoviral vectors at room temperature for a period of 20–24 hr has been described (O'Riordan *et al.*, 1999). What resulted was a virion that was described as being "superpegylated." The authors reasoned that complete saturation of the viral capsid with PEG would exponentially increase the ability of the virion to evade the immune response and allow for significant levels of gene expression, on readministration of the vector. In this study, PEGylation methods were developed that reach 70–100% completion within 1–2 hr with minimal loss in viral titer, making them an attractive addition to large-scale production processes.

Significant loss of titer with CCPEG can be attributed to the production of large viral aggregates due to extensive cross-linking of the polymer with viral capsids. This is manufactured as a mixture of two isoforms, one that will attach to proteins by a single amide bond, and another that is capable of forming two amide bonds with the target protein. Once aggregates were removed, the remaining viral suspension proved to be highly infectious and extremely stable under various storage conditions. The SSPEG preparation also experienced a significant drop in titer after conjugation for 1 hr at room temperature. No aggregation phenomenon was detected with this compound; however, loss in titer could be attributed to attachment of multiple SSPEG molecules to a single lysine residue as has been described previously (Matsuyama *et al.*, 1992). To maintain substantial viral infectivity with significant modification, a reaction time of 75 min, which modified 70% of available lysine residues and maintained titer, was selected. While this was the only protocol that did not completely modify the viral capsid, the resultant vector could efficiently evade neutralization by immune serum and maintained the highest titers at the various

storage conditions with respect to the other PEGylated preparations. These data indicate that complete modification of the viral capsid with this polymer may not be a strict requirement for production of highly efficient viral vectors.

Polyethylene glycol has been incorporated in protein formulations because of its ability to maintain protein conformation during the freezing step of lyophilization (Allison *et al.*, 1999). Incorporation of PEG in protein preparations can act as a double-edged sword, as it can maintain protein conformation while enhancing stability at low temperatures, but stabilizes the unfolded structure at elevated temperatures, which is useful for concentration and precipitation of proteins and viruses at ambient temperatures (Ingham, 1990; Polson, 1993). Both phenomena were displayed in our stability studies as MPEG, when added at a concentration similar to that employed in the PEGylation reaction, extended viral stability at 42 and 25°C beyond that of the unconjugated adenovirus, but viral titers were substantially lower than those of the PEGylated virions. Precipitates were eventually detected in these preparations, which significantly contributed to losses in transduction. However, addition of this excipient to adenovirus formulations at 4°C significantly enhanced stability beyond that of the native virus for up to 5 months because of its ability to maintain capsid assembly at this temperature. Glycerol is also added to formulations of biologics prior to freezing in order to maintain protein conformation during the freezing process (Nermut and Eason, 1985; Rowley, 1992). This excipient is also capable of replacing water molecules and prevents precipitation of formulation components during the freezing process. Addition of glycerol to PEGylated preparations did not affect the stability of the virus at −20°C. This result is encouraging as glycerol (and its associated toxicity) can be eliminated from preparations for clinical use. While each PEGylated preparation lost about 1 log of titer over 4 months, they were stored in the absence of any additional cryoprotectants. Addition of carbohydrates, surfactants, and other stabilizers will enhance stability and reduce loss in titer to negligible levels. It is also important to note that, as for any pharmaceutical compound, stability profiles can be concentration dependent and that stability profiles reported here may not correlate to Ad preparations stored at higher concentrations.

Stability profiles of PEGylated preparations at room temperature were quite impressive. TMPEG and SSPEG preparations showed negligible loss in infectivity for a period of 24 hr, which would allow for priority shipment of vector in the absence of dry ice. It is also important to note that these data were generated with vector stored in KPBS alone. Addition of excipients such as carbohydrates, surfactant, and other stabilizers could improve this further. The SSPEG preparation demonstrated a negligible loss of titer at 42°C for up to 8 hr. Thus, this preparation could survive exposure to extreme temperatures on shipping without significant loss of titer.

PEGylation of adenoviral vectors enhanced transduction efficiency when administered intratracheally or intravenously. This effect was somewhat unexpected as the majority of lysine residues that are present on the viral capsid are concentrated on the fiber and penton proteins, which are necessary for viral binding and entry into target cells (Adam *et al.*, 1977; Wickham *et al.*, 1993; Bergelson *et al.*, 1997). However, the new physical characteristics of the PEGylated viruses may contribute to the observed increase in viral transduction. Zeta potential measurements have shown that the adenovirus bears a significant negative charge on the capsid. Surface charge of viral vectors can significantly affect the level of transduction in various target tissues (Arcasoy *et al.*, 1997a; Fasbender *et al.*, 1997). It has been found that adenoviral transduction is inhibited due to static repulsion between the negatively charged sialic acid residues on the cell surface and the virus (Arcasoy *et al.*, 1997b). PEGylation effectively masks the groups responsible for this charge, producing an environment that would favor nonspecific interaction of the virus with the cell membrane. Particle size measurements of the final PEGylated preparations also revealed that each method produced a suspension of single viral particles that enhance the number of virions that come in contact with cell monolayers and, as a result, can increase transduction efficiency. Partition coefficients for the PEGylated virions indicate that the modified vectors have an increased affinity for hydrophobic environments that would allow for an increased ability to indiscreetly partition through cell membranes. Initial studies to assess the mechanism by which transduction efficiency of PEGylated adenoviruses is enhanced support this theory, as permeability of the PEGylated vectors across differentiated monolayers is significantly enhanced (data not shown).

In summary, a rapid method for PEGylation of adenoviral vectors was developed. All of the methods tested produced preparations that were stable at 4, 25, and 42°C with respect to unconjugated virions. However, SSPEG preparations could be produced with the least amount of effort and demonstrated superior stability profiles. Transduction efficiency of all PEGylated preparations was higher than that of the native virus *in vivo*.

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